Video Article

Isolating And Immunostaining Lymphocytes and Dendritic Cells from Murine Peyer's Patches

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Abstract

Peyer's patches (PPs) are integral components of the gut-associated lymphoid tissues (GALT) and play a central role in intestinal immunosurveillance and homeostasis. Particulate antigens and microbes in the intestinal lumen are continuously sampled by PP M cells in the follicle-associated epithelium (FAE) and transported to an underlying network of dendritic cells (DCs), macrophages, and lymphocytes. In this article, we describe protocols in which murine PPs are (i) dissociated into single cell suspensions and subjected to flow cytometry and (ii) prepared for cryosectioning and immunostaining. For flow cytometry, PPs are mechanically dissociated and then filtered through 70 µm membranes to generate single cell suspensions free of epithelial cells and large debris. Starting with 20-25 PPs (from four mice), this quick and reproducible method yields a population of >2.5 x 10^6 cells with >90% cell viability. For cryosectioning, freshly isolated PPs are immersed in Optimal Cutting Temperature (OCT) medium, snap-frozen in liquid nitrogen, and then sectioned using a cryomicrotome. Tissue sections (5-12 µm) are air-dried, fixed with acetone or methanol, and then subjected to immunolabeling.

Video Link

The video component of this article can be found at https://www.jove.com/video/50167/

Introduction

Peyer's patches (PPs) are macroscopic aggregates of organized lymphoid follicles present throughout the small intestine of humans and mice (**Figure 1**) and constitute the primary sites at which mucosal immune responses are initiated against dietary antigens, commensal bacteria, microbial pathogens, and oral vaccines [1-4]. Unlike other peripheral lymphoid tissues such as the mesenteric lymph nodes, PPs lack afferent lymphatics. As such, adaptive immune responses in PPs are driven in response to antigens derived from the intestinal lumen. The sampling of luminal antigens is accomplished the by the follicle-associated epithelium (FAE), which consists of both enterocytes and antigen-sampling cells known as M cells. Beneath the FAE, in the sub-epithelial dome (SED) region, lies a network of dendritic cells (DCs) intermingled with macrophages, B cells, and $CD4^+$ T cells [5-9]. At the core of each PP lymphoid follicle are follicular dendritic cells (FDCs) and a B cell-rich central germinal center, flanked by T cell-rich interfollicular zones. Antigen sampling by PPs results in the development of IgA+ B cell plasmablasts and $CD4^+$ effector and memory cells that seed the surrounding lamina propria and provide immunity to a wide range to mucosal invaders.

Dissecting the complex immunologic events associated with antigen sampling, processing, and presentation in PPs is a daunting task, considering that PP cells constitute only a tiny fraction of the total lymphoid cells in intestinal mucosa. To aid in the *in vitro* characterization of cells in this environment, we provide a protocol for preparing total mouse PP cells for flow cytometric and functional analysis, as well as a protocol for preparing PP cryosections for immunofluorescence microscopy and immunohistology. Our protocol for the isolation, characterization, and immunostaining of mouse PP cells is not novel *per se*, as evidenced by the fact that there are numerous references dating back more than 25 years that cite these techniques [5,6,9-11]. Rather, our protocol provides a streamlined (and visual) method for investigators collecting PPs for the first time. The techniques we describe are easily mastered and readily yield large numbers of cells with >90% cell viability. The cryosectioning protocol yields highly reproducible serial sections ideally suited for immunofluorescence staining and confocal imaging. Furthermore, our protocol complements two other recent *JoVE* articles. The first, by Fukuda and colleagues, describes the use of ligated ileal loop assays to assess the uptake of pathogenic bacteria by PP M cells [12]. The other, by Geem and colleagues, describes the isolation and characterization of DCs and macrophages from the mouse intestinal mucosa, but explicitly excludes PPs from their analysis [13].

Protocol

Animals were housed under conventional, specific pathogen-free conditions and were treated in full compliance with the Wadsworth Center's Institutional Animal Care and Use Committee (IACUC) guidelines.

1. Oral Gavage

1. (Optional) Gavage mouse strain of choice with antigen or microbes of interest using a 22 G x1.5-in. blunt-end feeding needle (Popper Scientific, New Hyde Park, NY). Delivery volumes should not exceed 400 µl per mouse.

2. Isolation of PP Cells for Flow Cytometry

1. Euthanize mice by CO_2 asphyxiation, according to institutional animal care and use committee (IACUC) guidelines.
2. Cleanse the abdomen with 70% ethanol or betadine prior to surgery. Perform a standard laparotomy that involves a single (1 cm) incision using surgical grade scissors along the midline beginning about 1.5 cm from the base of the rib cage. Expose the peritoneal cavity and identify the cecum. Snip the terminal small intestine at the ileal-cecal junction and gently remove the intestine in its entirety. Take care not to hyperextend the intestinal tissue as it is being removed from the peritoneal cavity.
3. Lay the small intestine on a bed of moist paper towels or Kimwipes. Wet the small intestine gently with saline to prevent tissue dehydration. Visually identify individual PPs located on the anti-mesenteric side of the intestine (**Figure 1**). Typically, a single mouse has 5 to 10 visible PPs that are evenly distributed from the duodenum (proximal) to ileum (distal). On average, four mice will yield 23 PPs.
4. Using curved surgical scissors, gently excise individual PPs and place them in cold Hank's Balanced Salt Solution (HBSS). **Note**, the scissors should be placed curve-side up just above the PP and then gently applied to the tissue. Excise only the PP (not surrounding tissue) and transfer to cold HBSS.
   Note: *All incubations and centrifugation steps from this point forward in Section 2 should be done at 4 °C to preserve cell viability.*
5. Transfer PPs into 5 ml Spleen Dissociation Medium and incubate for 15-20 min at 37 °C while vigorously shaking at 250 rpm. Longer incubation time will negatively affect cell viability.
6. To generate a single cell suspension, place PPs on a sterile (autoclaved) 70 µm nylon mesh cell strainer and forcibly grind the tissue into the mesh using the base of a plunger from a 1 cc syringe. Alternatively, sandwich PPs between two frosted sterile glass microscope slides (autoclaved in envelopes) and gently grind tissues with a back and forth motion. Use a sterile transfer pipette to transfer the cell suspension into a 5 ml Falcon tube.
7. Add EDTA to a final concentration of 1 mM to the cell suspension. Incubate on a rocker for 5 min at room temperature.
8. Decant cell suspension through a second 70 µm cell strainer to remove any remaining cellular aggregates or tissue debris. Collect cells in a 15 or 50 ml conical tube.
9. Subject cells to gentle centrifugation (5 min at 500 x g). Decant supernatant and resuspend cells in flow buffer, which is phosphate buffered saline (PBS) containing 1% fetal calf serum (FCS).
10. To determine cell number and viability, dilute cells 1:10 into trypan blue and visually inspect cells using a light microscope and a hemocytometer. Alternatively, a Countess cell counter (Invitrogen) or similar instrument can be used to automatically enumerate cell numbers and viability.
    Starting with 20-25 PPs, this protocol should yield >2.5 x 10^6 total cells. This equates to ~ 0.8-1.2 x 10^6 cells per mouse.

Antibody Labeling of Cells for Flow Cytometry

11. Dispense cells (10^5 per well) into wells of a 96-well round bottom plate.
12. Subject plate to gentle centrifugation (5 min at 1,000 x g), and decant supernatant by gently inverting the plate.
13. Resuspend cells in Fc block buffer and incubate on ice for 15 min. While there are a number of commercially available Fc block buffers (*e.g.* rat anti-mouse CD16/CD32, BD Biosciences), we simply use spent medium from a rat B cell hybridoma (ATCC 2.4.G2) that secretes a monoclonal IgG_1 against murine Fcγ receptors for this step.
14. Subject plate to gentle centrifugation (5 min at 1,000 x g) as above, and decant supernatant by gently inverting the plate.
15. Add fluorophore-conjugated antibodies directly to cell suspensions at desired dilution, and incubate on ice for 30 min with continuous rocking.
16. Subject plate to gentle centrifugation (5 min at 1,000 x g), and decant supernatant by gently inverting the plate.
17. Wash cells with 100 µl of flow buffer.
18. Centrifuge plate at 1,000 x g for 5 min, and decant supernatant by gently inverting the plate.
19. Resuspend cells 400 µl fixation buffer, which consists of 300 µl of flow buffer and 100 µl of 1% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgCl_2$ at pH 6).
20. Subject cells to flow cytometry using a FACSCalibur or equivalent. Analyze results using Cell Quest Pro software version 5.2.

3. Preparation of PP Cryosections

1. In advance of tissue collection, add Optimal Cutting Temperature (OCT) compound to 7x7x5 mm plastic base molds. Also prepare a pool of liquid nitrogen (>750 ml) in a dewar or ice bucket.
2. Euthanize mouse and perform a laparotamy, as described above. Remove intestine and place on wet paper towels.
3. To isolate PPs for cyrosectioning, cut 0.5 cm transverse segments of small intestine containing a single PP and transfer tissue to a Petri dish containing PBS. Gently rinse the lumen of these segments with PBS to remove unwanted fecal debris.
4. Place intestinal tissue segments vertically inside base molds containing OCT. Immerse the molds in liquid nitrogen and allow tissue to freeze completely (1-2 min). The color of the OCT will change from clear to white when the tissue is fully frozen. For a more gradual cooling (*e.g.* to reduce cracking of the OCT), immerse mold in a bath of isopentane cooled with liquid nitrogen vapors. **Note:** Wear appropriate safety goggles when working with liquid nitrogen.
5. Remove frozen base molds from the liquid nitrogen using forceps and allow the mold to warm at room temperature just long enough (~10-15 sec) to allow the edges of the OCT block to soften. To then remove the frozen block of OCT from the mold, invert the mold and press gently on the back to eject the block onto a clean 4 x 4 cm piece of aluminum foil. Immediately wrap the tissue in the foil and place in a labeled specimen bag stored in liquid nitrogen or on dry ice. Alternatively, transfer tissue in a freezer (<-20 °C). Use the tissue within a week, otherwise the blocks will become brittle with age.

Cryosectioning

6. Cryosection the tissue using a Leica CM3050S cryomicrotome or equivalent. The chamber temperature on the cryostat should be set to -21 °C.
7. Strive for sections that are 12-14 µm in thickness.
8. Collect sections onto Fisher SuperFrost Plus (or equivalent) glass microscope slides and inspect visually using a standard low power light microscope. If multiple sections are being collected on a slide, ensure that they are clustered together for downstream staining applications.
9. Store the slides at room temperature in a slide box and, ideally, use them within a few days.

Antibody Labeling of Cryosections and Confocal Microscopy Analysis

10. Immerse slides in acetone (or methanol) in tissue staining jar or racks for 2 min. Prolonged incubation may cause tissues to detach from the slide.
11. Transfer slides to new staining jar and wash 3 times with PBS-T (1X PBS with 0.05% Tween-20) for 3 min each.
12. Encircle the sections with an ImmEdge hydrophobic pen. This will ensure that reagents and antibodies will stay confined to that area during incubations.
13. Incubate slides in block buffer (2% goat serum in PBS) for 30 min at 37 °C in a humidified chamber, protected from light.
14. Incubate slides with Fc block buffer (see above) for 10 min at 37 °C.
15. Dip slides in PBS-T and dry area around sections using Kimwipes or other absorbent tissue. Take care not to touch the actual tissue sections.
16. Overlay tissue sections with primary antibody solution and incubate for 30 min at 37 °C in a humidified chamber. Primary antibody is typically diluted in block buffer to a final concentration of 20 µg/ml. The use of directly-labeled primary antibodies is desirable, because as many as three antibodies can be combined directly at this step. Directly-labeled antibodies also eliminate the need for additional antibody incubation steps.
17. Wash slides 3 times (5 min each) by immersion in PBS.
18. If necessary, incubate slides with relevant secondary antibodies for 30 min at 37 °C in a moisture chamber.
19. Wash slides 3 times (5 min each) by immersion in PBS.
20. Incubate slides for 4 min in 1% paraformaldehyde in PHEM buffer, as described above.
21. Rinse slides with PBS for 1 min.
22. Dry area around sections using Kimwipes or other absorbent tissue. Take care not to touch the actual tissue sections. Using a pipette or dropper, gently place a drop of Prolong Gold mounting medium directly onto the section. Apply a glass coverslip onto the mounting medium taking care to avoid bubbles. Use blotting paper to absorb any excess mounting medium.
23. Seal coverslips to microscope slides with commercial nail polish and allow to air dry.
24. View slides with a Leica TCS SP5 or equivalent confocal microscope.

Representative Results

Flow cytometric analysis of monodisperse suspensions of total PP cells reveals a clear distinction between good and poor cell preparations. In good cell preparations with over 80% viability, the vast majority of cells demonstrate high forward scatter (FSC), an indicator of high cell volume, and low side scatter (SSC), an indicator of low cell granularity (**Figure 2A**). In this experiment, we also intentionally prepared a "poor cell preparation" by incubating PP cells during the isolation steps at room temperature (instead of on ice) and, in the last step, incubating cells with PBS plus 1% FCS (instead of PHEM buffer). In these poor cell preparations, the majority of the cells exhibit low FSC and high SSC and are outside the indicated gate R1 (**Figure 2B**). These cells are likely undergoing apoptosis and/or necrosis.

Figure 3 demonstrates the use of a cocktail of up to four different fluorophore-conjugated monoclonal antibodies to discriminate among a variety of cell types in PP single cell suspensions. Labeling with cluster of differentiation (CD) markers CD19 and B220 reveal that B cells constitute ~60% of the gated PP cell population (**Figure 3A**). Specific T cell subsets can be easily enumerated by double labeling with antibodies against CD3 and CD4 (**Figure 3B**) or CD3 and CD8 (**Figure 3C**). $CD4^+$ and $CD8^+$ T cells constitute ~23% and ~9%, respectively of the total PP cells. DCs subsets labeled $CD11c^+$ (**Figure 3D**) and $CD103^+$ (not shown) can also be enumerated by this method. $CD11c^+$ DCs contribute to about 8% of the total gated population, which is roughly equivalent to 10,000 DCs per PP. This agrees exactly with the number of DCs observed by others [14].

Among the population of $CD11c^+$ cells approximately 4.7% were double positive for $B220^+$. Similar results are obtained when PP cells are collected from C57B/6 mice (**Figure 3E**).

"Poor" cell preparations can lead to highly misleading results, largely because dead or dying cells tend to bind antibodies non-specifically. As shown in **Figure 4**, the population of cells that stain positive for both the B cell marker (B220) and the T cell markers (CD3, panel A; CD4, panel B) can vary by more than 3-fold between a good and bad cell preparation. **Figure 4** shows an over representation of $B220^+$ and $CD3^+$ double-positive cells (Panel A), as well as $B220^+$ B cells and $CD4^+$ T cells double-positive cells (Panel B) in poor cell preparations. As mentioned above, the disparity in relative B and T cell numbers in good versus poor is likely due to non-specific binding of antibodies dying cells.

Our protocol also demonstrates that mouse PP cryosections are amenable to histopathologic analysis, as well as immunolabeling. **Figure 5** compares H&E staining of mouse PPs paraffin (panels A, B) and frozen sections (panel C). While the paraffin sections provide more resolution at the cellular level when stained by H&E, the light and dark zones of PP germinal centers are more easily demarcated in cryosection sections (**Figures 5A-C**). H&E-stained cryosections are useful for side-by-side comparison with immunolabeled cryosections. A typical PP cryosection stained with anti-CD3 antibodies to label the T cells, anti-CD11c antibodies to highlight DCs and anti-B220 antibodies to highlight B cells is shown in **Figure 6**.

Figure 1. Mouse Peyer's patches. Image of a freshly excised mouse small intestine with three visible PPs (white arrows). The PPs appear as blister-like structures (5 x 5 mm) on the anti-mesenteric side of the intestinal serosa.

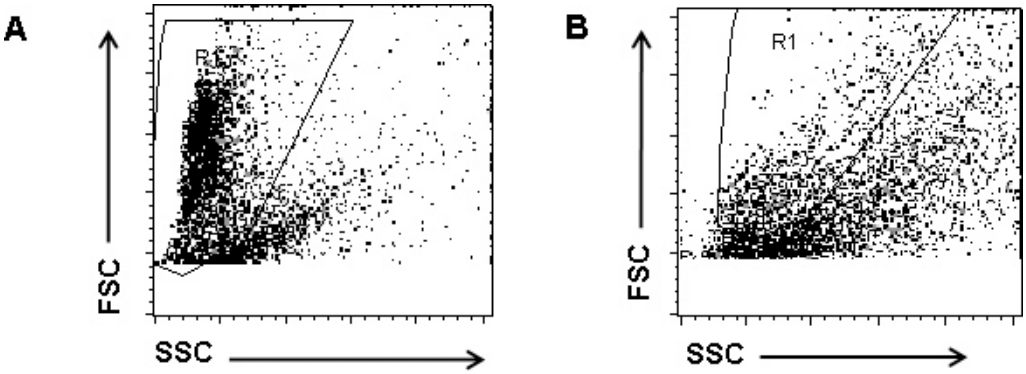

Figure 2. Total PP cells analyzed by flow cytometry. A monodisperse suspension of total PP cells was subjected to flow cytometry using a BD FACSCallibur. **(A)** Example of a good cell preparation. The vast majority of cells demonstrate high forward scatter (FSC), an indicator of high cell volume, and low side scatter (SSC), an indicator of low cell granularity. These cells are boxed in R1. Cells with low FSC and high SSC, located outside of R1 gate, are considered dead or dying cells. **(B)** Example of a poor cell preparation, in which the majority of the cells are outside the gate R1 due to low FSC and high SSC.

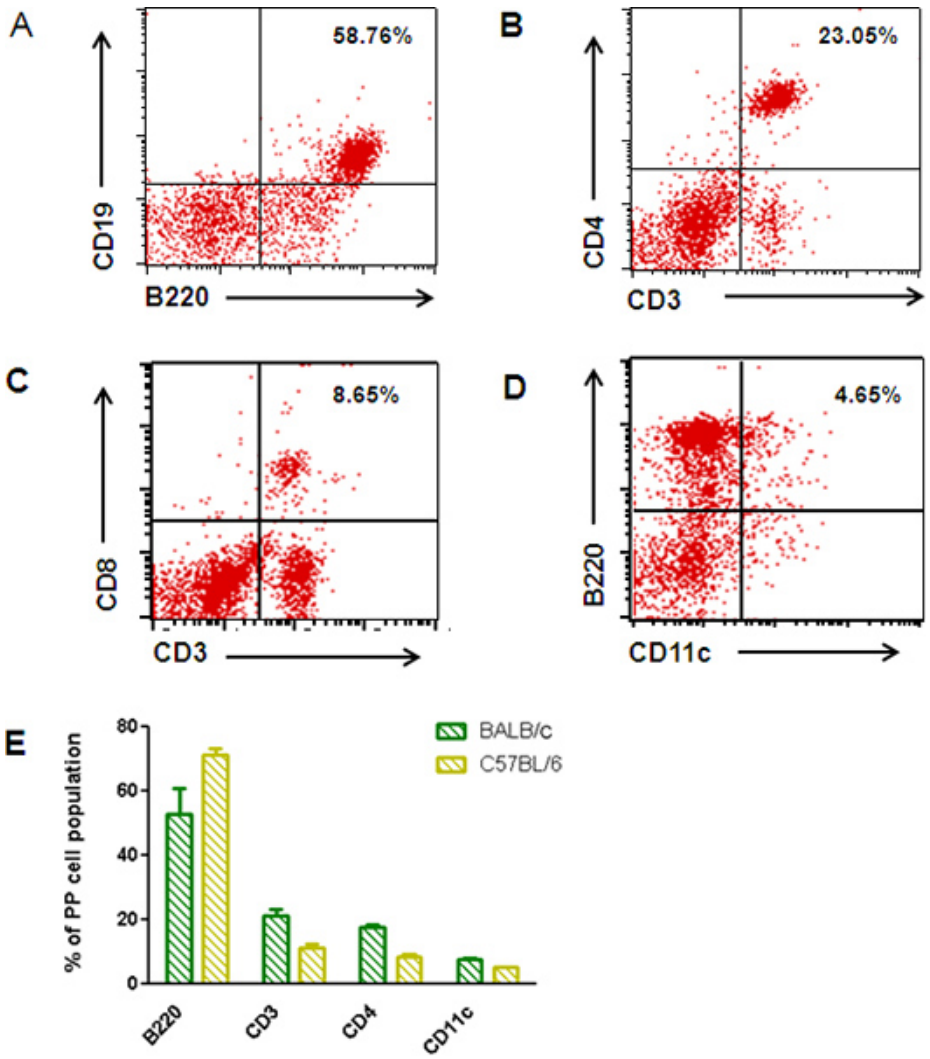

Figure 3. Representative flow cytometric analysis of PP lymphocytes. Monodisperse suspensions of total PP cells incubated with fluorophore-conjugated primary antibodies and then subjected to flow cytometry. **(A)** CD19 and B220 labeling of PP B cells. **(B-C)** Double-labeling of total cell populations with CD3 and CD4 **(B)** or CD8 **(C)** to enumerate specific T cell subsets. **(D)** Double-labeling of total cell populations for B220 (B cell marker) and CD11c (DC marker). **(E)** Comparison B220, CD3, CD4, and CD11c staining on PP cells from BALB/c and C57B/6 mice.

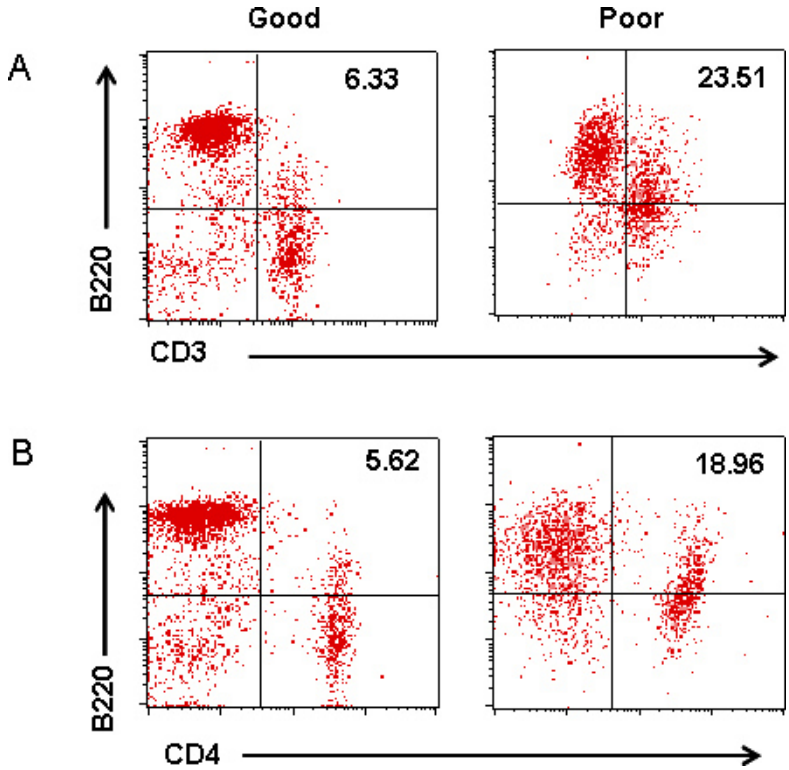

Figure 4. Misleading flow cytometry data as a result of poor PP cell preparations. Good (left panels) and poor (right panels) PP cell preparations were stained with **(A)** antibodies to B220 and CD3 to differentiate B and T cell populations or **(B)** antibodies to B220 and CD4 to differentiate B cells from a subset of T cells. There are generally very few cells of cells that stain positive for B220 and CD3 or CD4, as shown in the left panels. In contrast, in poor cell preparations double-positive cells constitute almost 20% of the total cells. See text for more details regarding what constitutes a "poor" cell preparation.

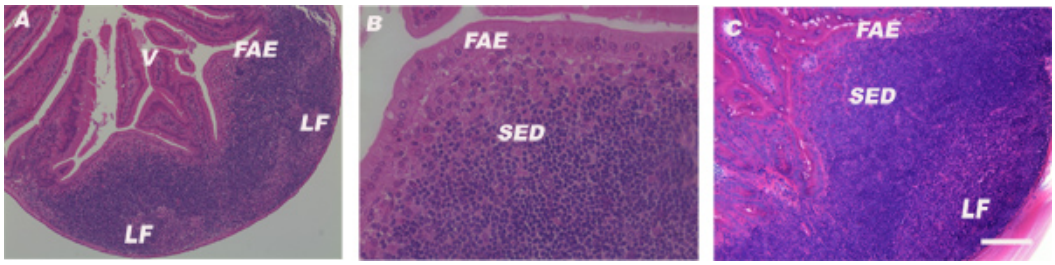

Figure 5. Representative H&E-stained PP sections. Paraffin-embedded sections **(A,B)** or cryosections **(C)** of ileal PPs were stained with H&E and visualized by light microscopy. **Note** that the light and dark zones of the lymphoid follicles are easily demarcated in the cryosections, as compared to the paraffin sections. Abbreviations: V, villous; FAE, follicle-associated epithelium; SED, sub-epithelial dome; LF, lymphoid follicle.

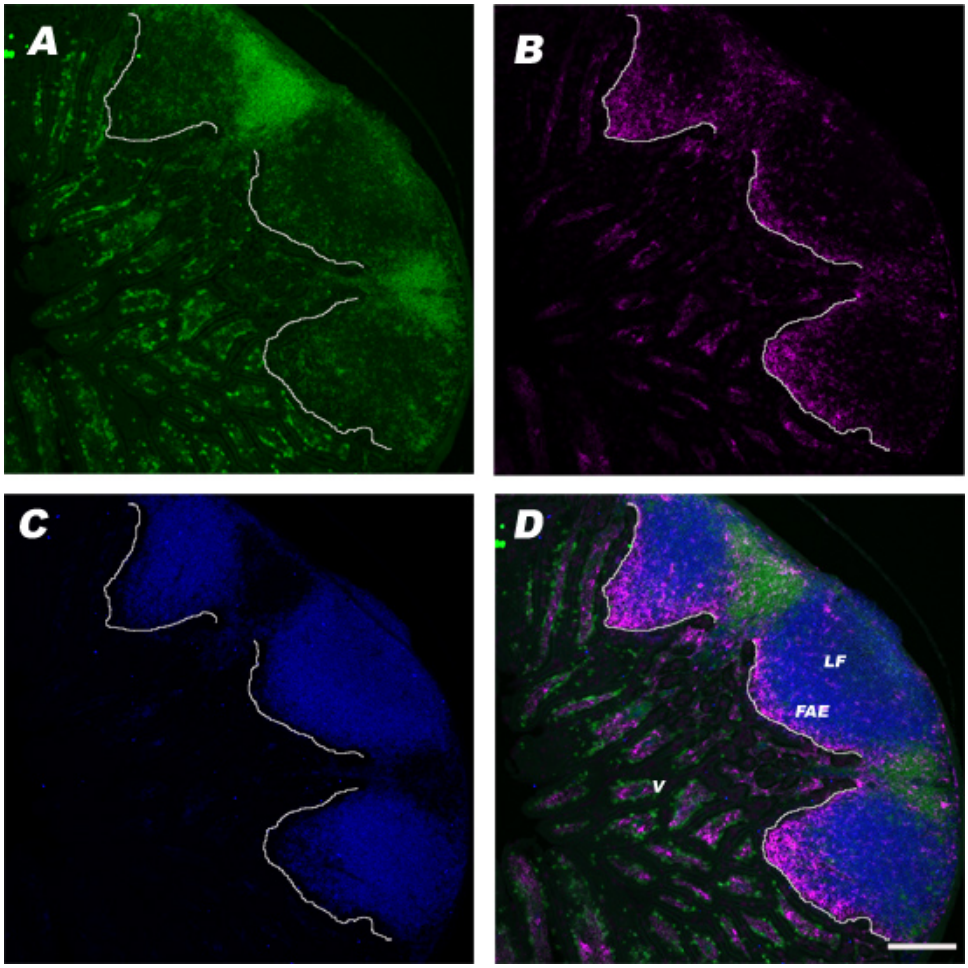

Figure 6. Immunofluorescent labeling of murine PP cryosections. Freshly cut tissue sections of a single mouse PP were air-dried, acetone-fixed, and stained with **(A)** FITC-conjugated anti-CD3 antibodies to label the T cells in the inter-follicular regions and lamina propria; **(B)** PE-conjugated anti-CD11c antibodies to highlight DCs in the SED; and **(C)** APC-conjugated anti-B220 antibodies to highlight B cells. **(D)** A composite of images in panels A-C generated using Fiji software. Scale bar is ~100 µm.

Discussion

In this article, we have provided parallel protocols for preparing PP single cell preparations for flow cytometric and functional analysis and cryosections for immunostaining. Both methods are highly reproducible and readily accessible, provided a flow cytometer and cryostat are available. For first time investigators it should be pointed out that when compared to the spleen, total cell yields from PPs are relatively meager. Nonetheless, the protocol we outline generally yields between 0.8-1.2 x 10^6 total PP cells from a single mouse. Scale-up is possible, although we typically do not pool more than 20-25 PPs, because, in our experience, aggregation occurs and cell yields (and viability) decline greatly. On the other hand, we do not recommend using fewer than 15 PPs for this protocol, as a critical mass is necessary to successfully carry the cells through the entire isolation procedure. If maximal cell viability is a priority for downstream functional assays, we recommend that a small sample of isolated cells be subjected to propidium iodide staining to allow for more accurate gating the viable pool of cells from the total PP cell population. Finally, it should be noted that our method is amenable to cell sorting applications if a specific cell subset is desired for adoptive transfer or *in vitro* analysis, for example.

The collection of PPs for cryosectioning is relatively straightforward, although the actual sectioning of PP tissues can be challenging. It is imperative, for example, that PP tissues are properly oriented in molds (i.e. perpendicular to the base of the mold) to ensure that true transverse sections are obtained. We propose snap-freezing PP tissues and then subjecting the cryosections to precipitating fixatives like acetone or methanol to preserve antibody reactivity ("antigenicity"), even though precipitating fixatives result in a decrease in overall cellular and sub-cellular resolution. If preserving antigenicity is not a particular concern, then we recommend the use of cross-linking fixatives like paraformaldehyde (PFA), which result in better preservation of cellular and sub-cellular structures. Indeed, PFA can be used to fix tissues prior to cryosectioning. Simply immerse freshly excised PPs in copious amounts of 4% PFA for >2 hr, wash tissue with PBS to remove excess fixative, equilibrate tissue in sucrose (15%) if desired, and then embed in OCT, as described above. The tissue can then be cryosectioned, but must be blocked with glycine solution (0.1 M in PBS for 15 min) to quench residual reactive PFA prior to immunostaining.

Finally, while we have described protocols for immunofluorescent labeling of PP cryosections, these same sections are amenable to immunohistochemistry (IHC) using commercially available IHC kits (*e.g.* Vector Labs, Burlingame, CA). For IHC, it is essential to include a

peroxidase blocking or quenching step in the protocol, as endogenous peroxidases are highly prevalent in the intestinal mucosa. Others have noted that for IHC, cardiac perfusion of the mice with 4% paraformaldehyde in PBS improves tissue preservation.

In summary, we have provided parallel and complementary protocols for quantitative and functional analysis of murine PP cells, including lymphocytes and DCs. The preparation of single cell suspensions enables quantitative and functional *in vitro* analysis of the major cell types in the PPs, while immunolabeling of cryosections provides information regarding the localization of specific cell types, as well as cell-cell interactions that exist *in situ*. The primary limitation of both these approaches is that neither represents "real time" visualization of PP cell-cell interactions. For the moment at least, PPs have proven impervious to intravital imaging, a technique that has revolutionized understanding of lymphocyte dynamics in peripheral lymph nodes. Until the technical barriers limiting intravital imaging of PPs are overcome, the protocols outlined in this article for preparing PP single cell preparations for flow cytometric and functional analysis and PP cryosections for immunostaining will remain the primary means of investigating and understanding the immunological functions of specific sub-populations of cells in PPs, the primary inductive sites of gut-associated lymphoid tissues.

Disclosures

No conflicts of interest declared.

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